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Non-Isotopic Detection of Hepatitis C Virus Quasispecies by Single Strand Conformation Polymorphism

Jung-Hun Lee,[1] Tobias Stripf,[1] W. Kurt Roth,[2] and Stefan Zeuzem[1]*

[1]*Medizinische Klinik II, Klinikum der Johann Wolfgang Goethe-Universität, D-60590 Frankfurt a.M., Germany*
[2]*Georg-Speyer-Haus, D-60596 Frankfurt a.M., Germany*

In patients infected with the hepatitis C virus (HCV), a heterogeneous population of viruses, so-called quasispecies exists in vivo. The hypervariable regions (HVR) within the second envelope gene (HCV-E2) show particularly highly intratypic variability and are considered to be the target of neutralizing antibodies. The aims of the study were to optimize a genotype-independent primer set for amplification of HVR-1 and to establish a sensitive SSCP analysis for rapid and non-isotopic detection of predominant serum HCV quasispecies. Using the optimized SSCP technique, changes of quasispecies composition were investigated in five chronically infected patients with HCV before and during interferon-α treatment. HCV genotyping was performed by sequence and phylogenetic analysis. In addition, serial viremia and serum alanine aminotransferase (ALT) levels were determined. The SSCP analysis was performed at two time points before and during interferon-α therapy, respectively. Four patients showed an alteration of the SSCP pattern during the first three months of interferon-α therapy, whereas in one patient the SSCP pattern changed before therapy and remained stable during treatment with interferon-α. The present approach for non-isotopic analysis of single strand conformation polymorphism provides a direct, rapid, and sensitive technique for detection of the heterogeneous population of HCV quasispecies of different genotypes. Using this test procedure, investigations of large cohorts of patients with chronic hepatitis C can be undertaken. ***J. Med. Virol. 53:245–251, 1997.***

KEY WORDS: Hepatitis C virus; hypervariable regions; HCV quasispecies; single strand conformation polymorphism; Interferon-α

INTRODUCTION

Patients infected chronically with hepatitis C virus (HCV) have a heterogeneous population of viruses, so-called quasispecies. The heterogeneity of HCV is due to the continuous and high replication rate in vivo, the low fidelity of the RNA-dependent RNA polymerase, and the immune surveillance of the host [Martell et al., 1992; Kato et al., 1993; Zeuzem et al., 1996]. The hypervariable regions (HVR) within the second envelope gene (HCV-E2) are considered to be the target of neutralizing antibodies. Sequence variations of HVR-1 during the natural course of chronic HCV infection was previously described [Kao et al., 1995]. Furthermore, the diversity of the HVR-1 quasispecies may influence the responsiveness to interferon-α therapy [Kanazawa et al., 1994]. Investigations of the HVR require, however, the time consuming technique of cloning and sequencing.

Single strand conformation polymorphism (SSCP) analysis is based on sequence dependent mobility shift of single stranded DNA (ssDNA) fragments in non-denaturing polyacrylamide gel electrophoresis [Orita et al., 1989]. Mutations within a defined DNA fragment cause different ssDNA conformations, thus leading to different electrophoretic mobility. The aims of the present study were to optimize a genotype-independent primer set for amplification of HVR-1 and to establish a sensitive SSCP analysis for a rapid and non-isotopic detection of predominant serum HCV quasispecies. The feasibility and reliability of the SSCP analysis was evaluated and changes of quasispecies composition were investigated in patients infected chronically with HCV before and during interferon-α treatment.

*Correspondence to Priv.-Doz. Dr. Stefan Zeuzem, Medizinische Klinik II, Zentrum der Inneren Medizin, Klinikum der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt a.M., Germany.

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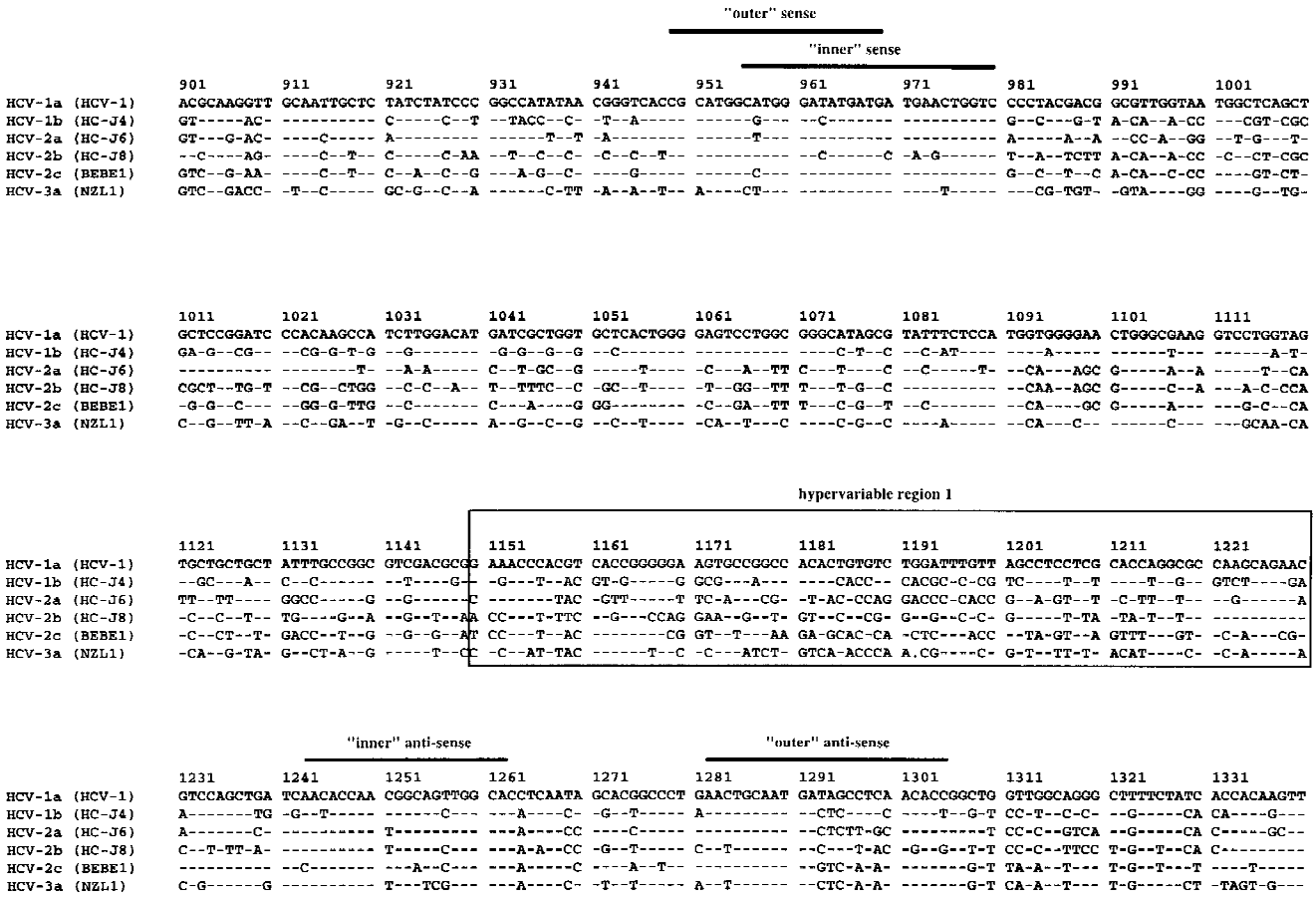

Fig. 1. Nucleotide sequence alignment of HCV prototype sequences for genotypes HCV-1a, -1b, -2a, -2b, -2c, and -3a within nucleotide positions 901 and 1340 (HCV-E1 and -E2 genes; numbering system according to Choo et al. [1989]) and location of an optimized "nested" primer set for amplification of HVR-1. Reference sequences: HCV-1 (HCV-1a, [Choo et al., 1989], EMBL accession number M62321), HC-J4 (HCV-1b, [Okamoto et al., 1992a], EMBL accession number D13558), HC-J6 (HCV-2a, [Okamoto et al., 1991], EMBL accession number D00944), HC-J8 (HCV-2b, [Okamoto et al., 1992b], EMBL accession number D10988), BEBE1 (HCV-2c, [Nakao et al., 1996], EMBL accession number D50409), NZL1 (HCV-3a, [Sakamoto et al., 1994], EMBL accession number D17763). Dashes indicate nucleotide positions identical to HCV-1 (HCV-1a). 5′-(sense) and 3′-(anti-sense) primer locations are shown as bold horizontal lines and the hypervariable region 1 is indicated by a rectangle.

MATERIALS AND METHODS

RT-PCR of the Hypervariable Region (HVR)-1

After extraction of HCV-RNA, cDNA synthesis was performed using random hexamer oligonucleotides (Boehringer Mannheim, Mannheim, Germany). HCV-cDNA was amplified using an optimized genotype-independent "nested" primer set ("outer" sense primer: 5′-CGCATGGCGTGGGACATGATG-3′; "outer" anti-sense primer: 5′-GGTGTGGAGGGAGTCATTG-CAGTT-3′; "inner" sense primer: 5′-GCTTGGGATAT-GATGATGAACTGGTC-3′; "inner" anti-sense primer: 5′-TGCCACCTGCCATTGGTGTT-3′) specific for the HCV-E1/E2 region (Fig. 1), which generated a PCR product of 307 base pairs (bp) (+956 to +1262 according to the numbering system of Choo et al. [1989]. The first round of "nested" PCR was performed by denaturation at 94°C for 60 sec, primer annealing at 55°C for 60 sec, and primer extension at 72°C for 60 sec. The amplification reaction was carried out for 25 cycles followed by a prolonged extension step at 72°C for 10 min. Two microliters of the first amplification product were transferred into the second PCR reaction mix containing the "inner" primer pair. The second round of amplification was performed for 35 cycles under the same conditions as described for the first round of "nested" PCR. The PCR product was analyzed on a 3% agarose-gel stained with ethidium bromide. Throughout the PCR application, measures to avoid contamination were strictly followed [Kwok and Higuchi, 1989].

PCR Cloning

HCV isolates of a chronically infected patient were amplified before and during interferon-α therapy using the HVR-1 "nested" primer set. The PCR products were cloned in the pGEM-T vector system (Promega, Madison, Wisconsin) and transformed into *Escherichia coli* JM 109 competent cells (Promega, Madison, Wisconsin). After overnight incubation at 37°C, white colonies

were transferred to 2 ml overnight culture. The plasmid was extracted by the alkaline lysis method [Maniatis et al., 1989] and the insertion was controlled by PCR using the HVR-1 "inner" primer pair. Ten clones of each PCR product with the correct insert were bidirectionally sequenced by an automat (Applied Biosystems 373A DNA Sequencer, Weiterstadt, Germany) using the HVR-1 "inner" primers according to the instructions of the Dye Deoxy™ Terminator protocol (Applied Biosystems, Weiterstadt, Germany).

Asymmetric PCR of the Clones

The PCR was carried out using asymmetric proportions of "inner" sense and anti-sense primers. For the sense PCR the reaction mixture contained 1.5 pmol of "inner" sense and 0.15 pmol of "inner" anti-sense primer and vice versa for the anti-sense PCR. The amplification conditions were as follows: a denaturation step at 94°C for 60 sec, an annealing step at 55°C for 60 sec, and an extension step at 72°C for 60 sec. The amplification reaction was carried out for 35 cycles.

Single Strand Conformation Polymorphism Analysis

SSCP analysis was undertaken by the Phast system™ (Pharmacia, Freiburg, Germany). Two microliters of the HVR-1 PCR product were mixed with two microliters of 94% formamide, denatured at 90°C for 2 min, and chilled immediately on ice. Four microliters of the denatured PCR product were applied on a 12.5% non-denaturing polyacrylamide minigel (Pharmacia, Freiburg, Germany) and the electrophoresis was performed at 15°C for 2 hrs (150 V for 300 Vh). The DNA bands were subsequently visualized by silver staining and photographed for documentation.

HCV Genotyping

HCV genotyping according to Simmonds' classification [Simmonds et al., 1993] was carried out by sequence and subsequent phylogenetic analysis of the nonstructural-5B region as described previously [Zeuzem et al., 1995].

HCV Quantification

HCV-RNA in serum was quantified by reverse transcription-polymerase chain reaction (RT-PCR) using a modified internal standard [Rüster et al., 1995]. The modification of the RNA standard consisted of an exchanged 25 base segment in a 256 base 5′-noncoding HCV-RNA fragment that was generated by site-directed mutagenesis by overlap extension using PCR. Detection of the coamplified mutant and the wild-type HCV amplification product was performed after denaturation and subsequent hybridization with sequence-specific biotinylated oligonucleotides. The sensitivity of the quantification system was 1×10^3 molecules per milliliter serum and the coefficient of variation was found to be 6.2% [Roth et al., 1996].

RESULTS AND DISCUSSION

The universal "nested" primer set used for HVR-1 PCR was designed by comparison of published nucleotide sequences of different HCV genotypes (Fig. 1). After optimizing the PCR conditions, a serum panel of 48 patients chronically infected with HCV genotypes 1, 2, 3, and 4 was used to assess the amplification efficiency of the HVR-1 PCR using this primer set. In this test panel, PCR amplification of 29/31 of HCV-1 samples (94%), 5/6 of HCV-2 samples (83%), 9/9 of HCV-3a samples (100%), and 2/2 of HCV-4 samples (100%) was achieved. The respective amplification efficiency in this panel using primers described previously [Enomoto et al., 1994] were 23%, 33%, 0%, and 0%, for genotypes HCV-1-4, respectively.

The HVR-1 PCR product of a HCV-1b isolate from a chronically infected patient was cloned before and three months after initiation of interferon-α therapy (3 MU thrice per week subcutaneously). Ten clones each were sequenced subsequently to determine the serum quasispecies composition. Before initiation of interferon-α therapy, four of ten clones (C1–C4) had identical sequences whereas the other six clones (C5–C10) differed in several nucleotide positions within the amplified region (Fig. 2A). Three months after initiation of therapy six of ten clones (C13, C15–C19) revealed different sequences, while only one clone (C20) showed the sequence of the previously predominant clone and 3 others the sequence of a previously minor clone (C11, C12, and C14) (Fig. 2A). These changes in quasispecies composition are reflected by changes of the (major) SSCP bands (Fig. 2B). For evaluation of the method, SSCP analysis was performed with clones C12, C16, C17, C18, C20, and the serum HVR-1 PCR product to relate single clones to respective SSCP bands. As shown in Figure 3, also minor quasispecies (C16, C17, C18, C20 present each in 1 of 10 clones) were detectable by the SSCP technique, suggesting a sensitivity of the assay of approximately 10%. This sensitivity was confirmed in similar experiments using additional samples (data not shown). The SSCP band pattern from an individual clone was highly reproducible (data not shown). Thus, the conformation of single stranded HCV-cDNA fragments of HVR-1 appears to be stable under these conditions. The sense and anti-sense strands generated by asymmetric PCR revealed different mobility in non-denaturing polyacrylamide gel electrophoresis. As shown in Figure 4, the sense strand migrated slower (upper band) compared to the anti-sense strand (lower band).

The resolution of the minigel system may not allow to infer the presence of individual clones. As shown in Figure 3, the position of individual strands may overlap in the total SSCP pattern. Furthermore, nucleotide variations outside the defined hypervariable region 1 (see Fig. 2A) will contribute to the SSCP pattern. Changes occuring in the region encoding for the E1 membrane anchor region will most likely not result from immune pressure of the host. As shown in Figure

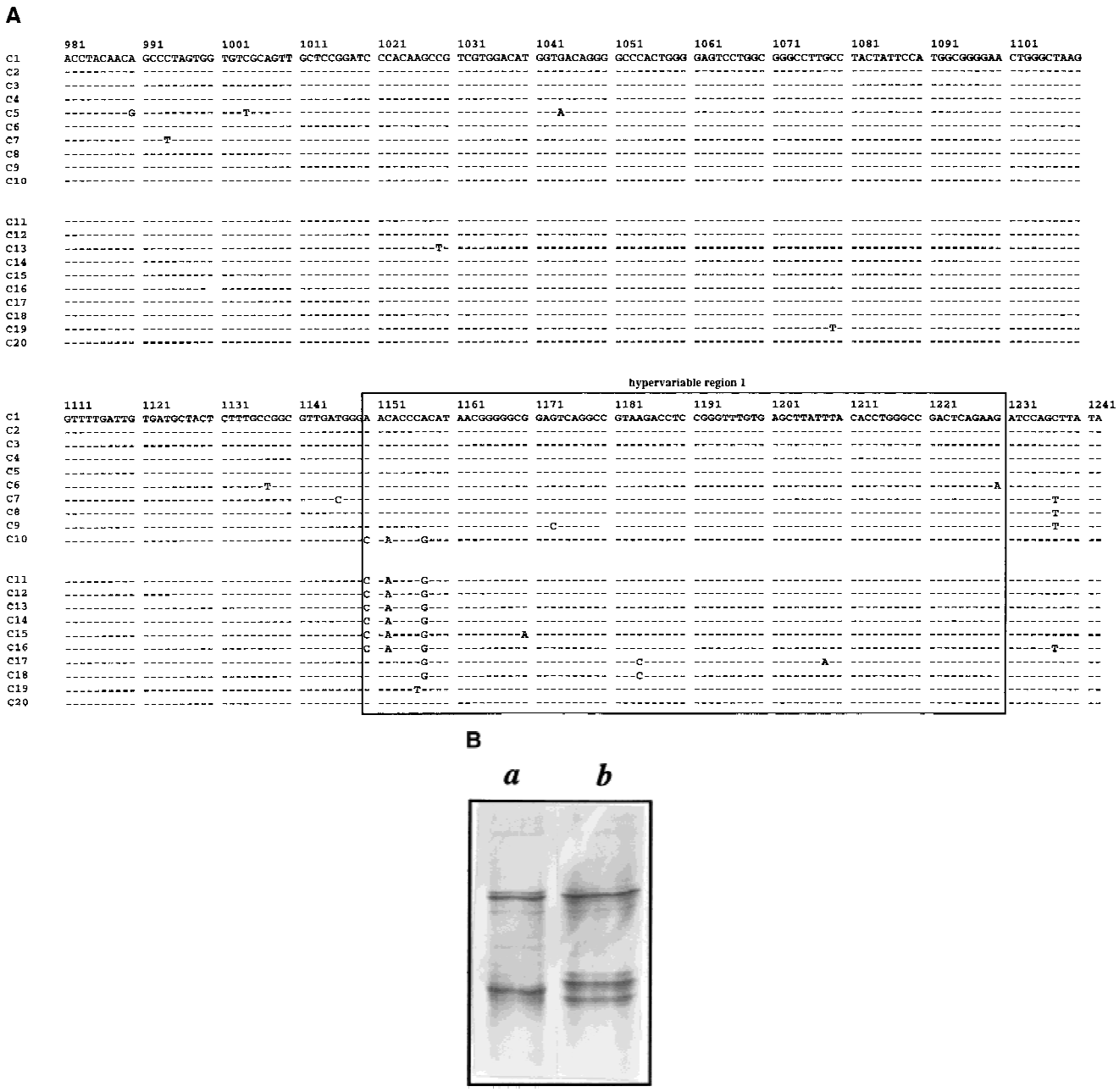

Fig. 2. Nucleotide sequences of HVR-1 clones detectable in serum before and during interferon-α therapy and SSCP analysis of serum HVR-1 PCR products. (A) Nucleotide sequences of 10 hypervariable region 1 (HVR-1) clones detectable in serum before initiation (C1–C10) and after three months of interferon-α treatment (C11–C20). Dashes indicate nucleotide positions identical to the predominant sequence in serum before initiation of interferon-α therapy. The predominant sequence in serum before treatment is indicated in bold letters and the HVR-1 (81 nucleotides; positions 1150–1230; numbering system according to Choo et al. [1989]) by a rectangle. (B) SSCP analysis using serum HVR-1 PCR products. Lane *a* corresponds to the PCR product before initiation and lane *b* to the PCR product three months after initiation of interferon-α therapy.

2A sequence analysis of the clones revealed only few additional mutations outside the HVR-1 region which did not substantially affect the relative variability of the quasispecies population before and during interferon-α therapy. The amplification efficiency of primers directly flanking the HVR-1 is highly insufficient and therefore not practicable. SSCP should therefore be used to investigate whether the quasispecies distribution has remained constant or whether it has evolved. Previous reports suggested that the number of SSCP bands allows quantification of (major) quasispecies [Enomoto et al., 1994; Gonzàles-Peralta et al., 1996]. Such interpretations, however, are questionable and require further comparative evaluation together with cloning and sequencing data.

In contrast to primers described previously for HCV-

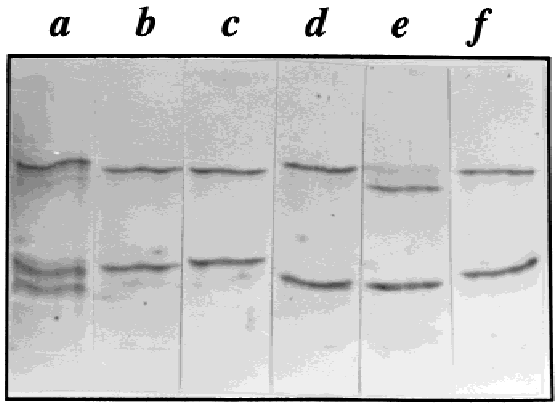

Fig. 3. Comparison of SSCP pattern of serum HVR-1 PCR product with the SSCP pattern of plasmid HVR-1 clones. Lane *a* displays the SSCP pattern of serum HRV-1 PCR product, lanes *b*, c, *d*, *e*, and *f* show clones C12, C16, C17, C18, and C20 as described in Figure 2A, respectively.

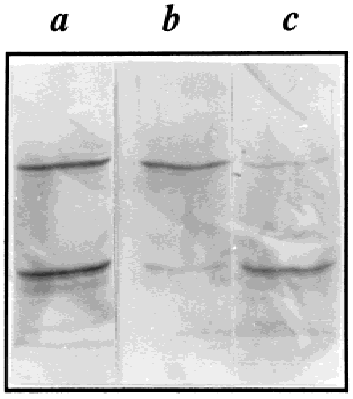

Fig. 4. Comparison of SSCP pattern of plasmid HVR-1 PCR product with the SSCP pattern of asymmetric PCR products. Lane *a* displays the plasmid HVR-1 PCR product amplified with 1.5 pmol of "inner" sense and anti-sense primer. Lane *b* corresponds to sense PCR product with 1.5 pmol of "inner" sense and 0.15 pmol of "inner" anti-sense primer, and lane *c* to anti-sense PCR product with 0.15 pmol of "inner" sense and 1.5 pmol of "inner" anti-sense primer.

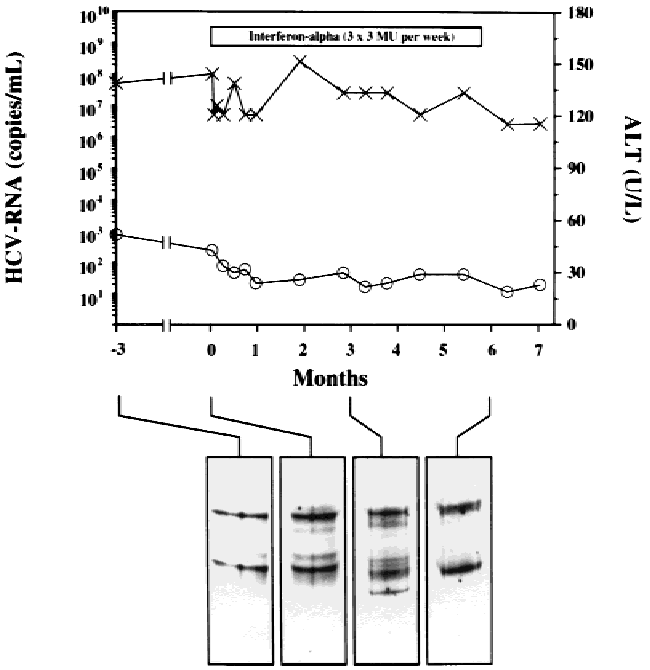

Fig. 5. SSCP analysis of a representative patient with chronic hepatitis C treated with interferon-α. The patient was infected with HCV-1b and treated with 3 million units interferon-α subcutaneously thrice per week for six months. SSCP analysis was performed before (−3 and 0 months) and during interferon-α therapy (3 and 6 months). Serial serum alanine aminotransferase levels (ALT) (○) and HCV-RNA levels (x) were determined as described in Methods.

E2 PCR, which considered only a subgroup of HCV genotypes (HCV-1b and -2) [Enomoto et al., 1994], HVR-1 amplification of European HCV-1, -2, -3, and -4 isolates could be achieved using the optimized "nested" primer set described in the present study. Since this primer set for the HCV-E2 region has a large spectrum for HCV genotypes with nucleotide sequence differences of 30–40%, we anticipate that these primers have no or only minimal preselective effects on HCV quasispecies (nucleotide sequence differences of less than 5%).

Using the optimized HCV-E1/E2 PCR, HVR-1 amplification products of five patients with chronic hepatitis C were investigated by single strand conformation polymorphism analysis before (−3 and 0 months) and during interferon-α therapy (3 and 6 months). Three patients were infected with HCV-1b, the other two patients with HCV-1a and HCV-3a, respectively. In all patients serum ALT levels were elevated more than twice the upper limit of normal (<23 U/L) before initiation of interferon-α treatment (range: 47–174 U/L). None of the patients showed a sustained response, only the patient infected with HCV-3a revealed a transient decline of HCV-RNA serum levels. Four patients had a stable SSCP band pattern before initiation of therapy but alterations of the SSCP pattern during the first three months of interferon-α therapy, whereas in one patient the SSCP pattern changed already before therapy and remained constant thereafter. During further therapy the quasispecies population as assessed by SSCP of the HVR-1 amplification products remained constant in three patients. In the two remaining patients additional alterations of the SSCP pattern were observed at 6 months of treatment. In one patient infected with genotype HCV-3a the change of the SSCP pattern occurred after a transient decline of serum HCV-RNA levels in the first 3 months of interferon-α therapy. In the remaining four patients alterations of the quasispecies population were not associated with changes in hepatitis C viremia. In Figure 5 the SSCP band pattern, hepatitis C viremia, and serum alanine aminotransferase levels of a representative patient before and during interferon-α therapy is shown.

In this study, changes of the quasispecies population appear to be more pronounced after initiation of interferon-α therapy than during the natural course of the disease. The HVR-1 glycoprotein within the envelope

region may function as a target to circulating antibodies [Kato et al., 1992]. Interferon-α has antiviral and immunomodulatory effects and may eliminate predominant HCV species [Zeuzem et al., 1996]. Thereby, the previous quasispecies composition could change and minor or new resistant quasispecies may become predominant [Okada et al., 1992; Kanazawa et al., 1994]. This phenomenon was observed in one patient, where the previous minor clone C10 (present in 1 out of 10 clones before interferon-α therapy) became dominant (3 of 10 clones) three months after therapy (Fig. 2A). Changes within the quasispecies population are reported to also occur during the natural course of the disease [Kumar et al., 1993], particularly in patients with high aminotransferase levels [Kurosaki et al., 1993]. In the present study, however, the patient with an alteration of the SSCP pattern before interferon-α treatment, showed only a moderate elevation of serum ALT levels (52 U/L versus 114 ± 51 U/L in the four patients with no changes in the SSCP pattern). Recently, an association of breakthrough and relapse phenomena with the development of new escape mutants detectable in serum during or after interferon-α therapy was also observed [Weiner et al., 1992; Okada et al., 1992]. Taken together, evidence has accumulated that changes in HCV quasispecies occur during the natural course of disease and that these alterations are enhanced during interferon-α therapy.

In conclusion, the present approach for non-isotopic analysis of single strand conformation polymorphism provides a direct, rapid, and sensitive technique for detection of the heterogeneous population of HCV quasispecies. Nevertheless, there are some general limitations of the technique: (1) despite different nucleotide sequences, clones may have overlapping migration pattern, (2) therefore the number of SSCP bands does not necessarily reflect the number of (major) variants, (3) mutations within the amplified region but outside the defined HVR-1 will contribute to the SSCP pattern, and (4) the SSCP pattern cannot distinguish between silent mutations and mutations leading to amino acid exchanges. Using this test procedure, investigations of large cohorts of patients are conceivable (1) to test the hypothesis that new dominant quasispecies arise during antiviral therapy which already preexisted as a minor population in the pretreatment phase, (2) to study whether the alteration of the quasispecies population is more pronounced during antiviral therapy than during the natural course of disease, and (3) to evaluate the possible occurrence of resistant quasispecies as a cause of breakthrough phenomena during antiviral therapy.

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